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CONTRIBUTION TO THE PHYLOGENY OF THE PANGASIIDAE BASED ON MITOCHONDRIAL 12S RDNA

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ABSTRACT

Catfishes are generally one of the economically important groups of fresh and brackish water fishes in the world. In many countries, they form a significant part of inland fisheries, and several species have been introduced in fish culture. Judging from literature, the main constraint to cultivate wild species and to optimise the production of pangasiid catfishes is due to the poorly documented systematics of this family. In the present contribution, the phylogenetic relationships within Pangasiidae are studied to contribute to a better insight in their taxonomy and evolution. The genetic relatedness is inferred using mitochondrial 12S rDNA gene sequences. To resolve the phylogenetic position of *Laides* in this group of catfish, five genera of Asian and African Schilbeidae are also considered. The results showed that a species group (complex) could be clearly seen in the genetic tree. *Pangasius* is more derive than the other genera. By using approximate molecular clock/evolutionary calibration from mitochondrial gene, a new episode of speciation for the family marked explosive radiation about 5-8 million years ago (mya). This adaptive radiation extended until the Late Pleistocene. Regarding the relationships between the Pangasiidae and Schilbeidae, two families show an allopatric distribution with slight overlap. The Pangasiidae occur mainly in Southeast Asia, while the Schilbeidae are seen mainly on the Indian subcontinent (including Myanmar) and Africa. It confirms the separation between Schilbeidae and Pangasiidae occurred in the Early Miocene.

[***Keywords***: Pangasiidae, evolution, biogeography, taxonomy, morphology, mitochondrial DNA, cytochrome b gene, 12 S rDNA gene, genetic distance]

INTRODUCTION

Advances in DNA sequencing technology over the past decade have been phenomenal (Hillis *et al.* 1990; Meyer 1993; Simon *et al*. 1994). The power of the technique has ensured that it has become one of the most utilized molecular approaches for inferring phylogenic history (Hillis *et al*. 1990). The primary attraction of nucleic acid sequencing relates to the fact that the characters (nucleotides) are the basic units of information encoded in organisms (the genome), and that the potential of informative data sets are immense.

Fish may contain more than 10^8 nucleotide pairs per haploid genome, although the number of independent characters that could be used in phylogenetic analysis is considerably lower. To use nucleotide sequence positions in the phylogenetic studies, orthologous sequences must be aligned. The number and size of orthologous sequences that can be aligned will differ depending on the level of comparison, but for most studies, systematically informative variation is essentially inexhaustible (Hillis *et al*. 1990).

Due to the lack of molecular data, considerable confusion has arisen in the systematics of pangasiid catfishes. This is illustrated by the genus *Laides* Jordan, 1919, placed either in the Pangasiidae (Roberts 1989) or in the Schilbeidae (Vidthayanon 1993). Following Mo (1991), the Pangasiidae are the sister group of the Schilbeidae.

In Mekong Delta, the aquaculture production of *Pangasius* exceeds significantly the production from capture of fisheries, showing the economically importance of their aquaculture in the global fisheries sector. Meanwhile, in Indonesia, although more than 10 pangasiid species have been listed, the only *Pangasius* cultured remained *Pangasianodon hypophthalmus*, introduced from Thailand. Of the 28 valid species, few have been reproduced successfully: *P. hypophthalmus* since 1966, and more recently several others including *Pangasianodon gigas*, *Pangasius bocourti*, and *Pangasius djambal* (Roberts and Vidthayanon 1991; Legendre *et al.* 2000).

In the present contribution, the phylogenetic relationships within the Pangasiidae are studied to contribute to a better insight in their taxonomy and evolution. For this purpose, the genetic relatedness

between 23 out of the 24 valid species of the genera *Pangasius* and *Helicophagus* will be inferred using mitochondrial 12S rDNA gene sequences. These data will be combined with results from previous studies based on cytochrome b/allozyme phylogenies (Pouyaud *et al*. 2000). To resolve the phylogenetic position of *Laides* in this group of catfishes, five genera of Asian and African Schilbeidae (*Clupisoma* Swainson, 1838; *Eutropiichthys* Bleeker, 1862; *Silonia* Swainson, 1838; *Pseudeutropius* Bleeker, 1862; and *Schilbe* Oken 1817) will also be considered.

MATERIALS AND METHODS

Origin and number of samples used are listed on Table 1. Fresh specimens collected from 1996 to 2000 were dissected on site and tissue samples (eye, muscle, and liver) were stored in liquid nitrogen for transfer to the laboratory. They were then stored at -20ºC until analysis in the molecular genetic laboratory in Pasar Minggu, Jakarta, Indonesia.

Mitochondrial analysis consisted in sequencing part of the 12S rDNA gene. One to five individuals were analysed for 22 species of Pangasiidae and 7 species of Schilbeidae. Hundred milligrams of muscle were used to isolate total genomic DNA using a hexadecylmethyl-ammoniumbromide extraction process (Doyle and Doyle 1987).

Primers used for the amplification of the mitochondrial gene included 12S light strand 5'-TTACACATGCAAGTCTCCGC-3' and 12S heavy strand GTTACGACTTGCCTCCCCTT-3' defined on the complete mitochondrial sequence of *Cyprinus carpio* (EMBL X61010; Chang *et al*. 1994). DNA amplification and sequencing were performed following the procedures developed in Pouyaud *et al*. (2000). To check sequence accuracy and to correct any ambiguity bases, both strands were sequenced using each one of the two initial PCR primers. Sequences of both strands were compared with each other and aligned using the sequence editor ESEE (version 3.1 s; Cabot and Beckenbach 1989). Comparison of absolute numbers of transitions and tranversions were calculated and plotted versus corrected genetic distances using Kimura's distance two-parameter method (Kimura 1980). Phylogenetic inference was based on the neighbour joining (Saitou and Nei 1987) method (NEIGHBOR program in PHYLIP; Felsenstein 1993) from Kimura's distances among species. The reliability of the topologies was assessed with bootstrapping on 1000 replicates (SEQBOOT and CONSENSE programs in PHYLIP; Felsenstein 1993).

RESULTS AND DISCUSSION

All material listed was sequenced (Table 1). For many taxa, the results showed single sequence (no intraspecific variation) except for *Pangasius polyuranodon* and *Pangasius kunyit* having different mitochondrial haplotype from distinct geographic populations. In the following section, genetic interrelationships of the Pangasiidae, genetic interrelationships between Pangasiidae and Schilbeidae, evolutionary process based on speciation peaks, systematic position of the genus *Laides*, and speciation process are discussed.

Genetic Interrelationships of the Pangasiidae

Seven hundred and thirty seven nucleotides encoding the internal part of the mitochondrial 12S rDNA gene were aligned for all pangasiid species (Table 2). In the 737-bp sequences, 123 (17%) sites were polymorphic and 72 of these (59%) were phylogenetically informative.

In a first step, the genetic differentiation within each species studied was assessed to confirm the validity of the species recognised by Roberts and Vidthayanon (1991), Pouyaud *et al*. (1999), Pouyaud and Teugels (2000), and Ng and Kottelat (2000). For this purpose, corrected pairwise Kimura's genetic distances were computed considering a population as the unit of analysis. A phylogenetic tree was obtained from the genetic distance matrix using the nearest neighbour joining method (data not shown). This dendrogram revealed that all populations analysed for a given species aggregated and constituted in all cases of a monophyletic group (bootstrap between 77% and 100%), which confirms the existence of 23 out of 24 species recognised or described previously. Except for *P. polyuranodon* and *P. kunyit*, genetic distances among conspecific populations were null or insignificant [*Pangasius macronema*: two nucleotidic substitutions between Chao Phraya (Thailand) and Mekong (Vietnam) populations; *Pteropangasius micronemus*: a unique substitution between Batang Rajang (Sibu, Malaysia) and Batang Hari (Sumatra, Indonesia) populations].

The average Kimura's distances within species revealed significant genetic differentiation among populations of *P. polyuranodon* and *P. kunyit* located in vicariant biogeographic areas. *P. polyuranodon* is composed of three distinct genetic groups of populations, respectively from East Kalimantan (Samarinda), the rest of Indonesia (Sumatra, Central Kalimantan, West Kalimantan, Sarawak), and Indo-

Table 1. Species identification, sampling origin, collection reference, sample size (n) analysed with mitochondrial DNA.

Species identification[1]	n	Location (site, river, country)
Pangasius djambal Bleeker, 1846	1	Jambi, Batang Hari, Sumatra, Indonesia
	1	Barito, Central Borneo, Indonesia
Pangasius rheophilus Pouyaud & Teugels, 2000	1	Tanjung Selor, Kayan, Bulungan, East Borneo, Indonesia
	1	Tanjung Redep, Berau, Bulungan, East Borneo, Indonesia
Pangasius bocourti Sauvage, 1880	1	Mekong, Vietnam
Pangasius nasutus Bleeker, 1863	1	Musi, Sumatra, Indonesia
	1	Palangkaraya, Kahayan, Central Borneo, Indonesia
	1	Kapuas, West Borneo, Indonesia
Pangasius conchophilus Roberts & Vidthayanon, 1991	1	Mekong, Vietnam
Pangasius larnaudii Bocourt, 1866	1	Chau Doc, Mekong, Vietnam
Pangasius sanitwongsei Smith, 1931	1	Chau Doc, Mekong, Vietnam
Pangasius pangasius Hamilton, 1822	1	Chandpur, Ganges, Bangladesh
Pangasius krempfi Fang & Chaux, 1949	1	Mekong, Vietnam
Pangasius kunyit Pouyaud et al., 1999	1	Sanga-sanga, Mahakam, East Borneo, Indonesia
	1	Pontianak, Kapuas, West Borneo, Indonesia
	1	Barito, Central Borneo, Indonesia
	1	Kinabatangan, Sabah, North Borneo, Malaysia
	1	Mekong, Vietnam
Pangasius polyuranodon Bleeker, 1852	1	Jambi, Batang Hari, Sumatra, Indonesia
	1	Barito, Central Borneo, Indonesia
	1	Kapuas, West Borneo, Indonesia
	1	Batang Rajang, Sarawak, North Borneo, Malaysia
	1	Mahakam, East Borneo, Indonesia
	1	Mekong, Vietnam
Pangasius macronema Bleeker, 1851	1	Mekong, Vietnam
Pangasius humeralis Roberts, 1989	1	Sintang, Kapuas, West Borneo, Indonesia
Pangasius lithostoma Roberts, 1989	1	Sintang, Kapuas, West Borneo, Indonesia
Pangasius kinabatanganensis Roberts & Vidthayanon, 1991	1	Sukau, Kinabatangan, Sabah, North Borneo, Malaysia
Pangasius nieuwenhuisii Popta, 1904	1	Mahakam, East Borneo, Indonesia
Pangasianodon hypophthalmus Sauvage 1878	1	Mekong, Vietnam
Pangasianodon gigas Chevey, 1930	1	Mekong, Thailand
Pteropangasius micronemus Bleeker, 1847	1	Batang Hari, Sumatra, Indonesia
	1	Indragiri, Sumatra, Indonesia
	1	Jombang, East Java, Indonesia
	1	Sibu, Batang Rajang, Sarawak, Malaysia
Pteropangasius pleurotaenia Sauvage, 1878	1	Chao Phraya, Thailand
Helicophagus typus Bleeker, 1858	1	Jambi, Batang Hari, Sumatra, Indonesia
Helicophagus leptorhynchus Ng & Kottelat, 2000	1	Mekong, Laos
Schilbe mandibularis Günther, 1867	1	Comoé, Ivory Coast, West Africa
Clupisoma garua Hamilton, 1822	1	Chandpur, Ganges, Bangladesh
Laides sinensis Huang, 1981	1	Chao Phraya, Thailand
Silonia silondia Hamilton, 1822	1	Chandpur, Ganges, Bangladesh
Eutropiichthys vacha Hamilton, 1822	1	Chandpur, Ganges, Bangladesh
Pseudeutropius brachypopterus Bleeker, 1858	1	Muara Bungo, Batang Hari, Sumatra, Indonesia
	1	Sintang, Kapuas, West Borneo, Indonesia
Laides hexanema Bleeker, 1852	1	Teluk Kuantan, Indragiri, Sumatra, Indonesia

[1]Species identification is based on Roberts and Vidthayanon (1991) and Pouyaud *et al*. (2000).

China (Vietnam and Thailand). The average genetic distances between populations from East Kalimantan and those from the rest of Indonesia or Indo-China (d = 0.0253) is equivalent to average distances between *Pangasius conchophilus* and *P. bocourti* or between *P. nasutus* and *P. larnaudii*. The genetic differentiation between populations from the rest of Indonesia and Indo-China is the smallest (d = 0.0126), but corresponds to the genetic differentiation between *P. micronemus* and *P. pleurotaenia* (d = 0.014). Similarly, three genetic groups are found within the species *P. kunyit*, respectively from Indonesia

Table 2. Sequence alignment (12S rDNA gene) between Pangasiidae and Schilbeidae.

P. polyuranodon Rest of Indonesia	AGCCCAAGAC GCCTTGCTAC GCCACACCCC CAAGGGAATT CAGCAGTAAT AAACATTAAG CCATAAGTGT AAACTTGACT TAGTTAGGGC CA-A-AAGGG TCGGTAAAAT TCGTGCCAGC CACCGCGGTT
P. polyuranodon Indo-China	G. . . . .
P. polyuranodon East Kalimantan	. . . . .
P. rheophilus	. . . .T. . . . .
P. djambal	. . . . . C . . . . . A. . . . .
P. bocourti	. . . . . C . . . . . A. . . . .
P. nasutus	. . . .A. . . . .
P. conchophilus	. . . .G. . . . .
P. pangasius	. . . . . .T . . . . .
P. kunyit Indonesia	. . . . . .T . . . . .
P. kunyit Vietnam	. . . . . .T . . . . .
P. kunyit North Borneo	. . . . . .T . . . . . .G.. . . . . .G. . . . .
P. sanitwongsei	. . . . . .C. . . . . A . . . . .
P. larnaudii	. . . . . T . . . . . T. . . . .
P. humeralis	. . . . .
P. nieuwenhuisii	. . . . .
P. kinabatanganensis	. . . . . .A. . . T. . . . .
P. lithostoma	. . . . . C. . . . .
P. macronema	. . . . . T . . . . .
P. micronemus	. . . .T. . . . .
P. pleurotaenia	. . . .T. . . . . C . . . . . T. . . . .
P. hypophthalmus	. . . . . .A.. T. . . . .
P. gigas	. . . . . .T . . . . . C ..G. . . . . .T T. . . . .
H. leptorhynchus	. . . . . .T . . . . . .C. . . . . .C. . . .
H. waandersii	. . . . . .T . . . . . .C. . . . . ..A..C. . . .
H. typus	. . . . . .G . . . . . .T. . . . .
S. mandibularis	T. . . . . T. . . . . A. . . .G. . .C ....C. . . . . T. . . . .
L. sinensis	. . . .-.... . . . . .A..T T.AT. . . . . C. . . . .
C. garua	. . . . . .T . . . . . T. . . . .T. . . . . .A..T T.AC. . . . .
E. vacha	. . . . . .C.. . . . .T. . . . . C . . . .A...A..T T.ATT-. . . . C. . . . .
S. silondia	. . . . . .C.. . . . .T. . . . . C . . . . . .A..T T.ATT. . . . .
L. hexanema	. . . . . T . . . . . T.. . . . .T. . . . . .A..T T.AT. . . . .T. . . . .
P. brachypopterus	. . . . . .T.. . . . . .T. . . . . T . . . . . T. . . . .

Table 2. (continued).

P. polyuranodon Rest of Indonesia	ATACGAAAGA	CCCTAGTTGA	TAGCTACGGC	GTAAAGGGTG	GTTA-AG-GG	ACA--TA-AA	AATAAAGCTA	AAGATCCTCT	AAGCCGTCAT	ACGCACTCCG	AGGACACGAA	ACCCCAACAC	GAAAGTAGCT
P. polyuranodon Indo-China	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . G	. T . . . . G . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	G	. . . . .
P. polyuranodon East Kalimantan	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. T . . . C - T . .	. . . . .	. . . . .	. . . . . G	. . . . .	. . . . .	. . . . .	. . . . .
P. rheophilus	. . . . .	. . . . .	. . A	. . . . .	. . . . . A	. T	. . . . .	. . . . C	. . . . .	. . . . .	. . . . T	G	. . . . .
P. djambal	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . A . .	. . . . . G	. . . . CT	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. bocourti	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . A . .	. . . . .	. . . . CT	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. nasutus	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . A	. T . . . . AT .	. . . . .	. . . . C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. conchophilus	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . A	. . . . . A . .	. . . . .	. . . . C	. . . . .	. . . . .	. . . . T	G	. . . . .
P. pangasius	. . . . .	. . . . .	. . . . . G	. . . . .	. . . . .	. T	. . . . .	. . . . C	. . . . .	. . . . . T	. . . . .	G	. . . . .
P. kunyit Indonesia	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . C	. . . . .	. . . . .	. . . G	. . . . .	. . . . .
P. kunyit Vietnam	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . C	. . . . .	. . . . .	. . . G	. . . . .	. . . . .
P. kunyit North Borneo	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . C	. . . . .	. . . . .	. . . G	G	. . . . .
P. sanitwongsei	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . C	. . . . .	. . . . . C . . .	. . . . .	. . . . T	. . . . .
P. larnaudii	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . A	. . . . .	. . . . . T	. . . . C	. . . . .	. . . . .	. . . G	. T . . T	. . . . .
P. humeralis	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . A	. T	. . . . .	. . . . .	. . . . . T . .	. . . . .	. . . . .	G	. . . . .
P. nieuwenhuisii	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . A	. T	. . . . .	. . . . .	. . . . . T . .	. . . . .	. . . . .	G	. . . . .
P. kinabatanganensis	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. T	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. lithostoma	. . . C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . G .	. . . . .	. . . . C	. . . . .	. . . . .	. . . G	. . . . . T	. . . . .
P. macronema	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. T . . . . G . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	G	. . . . .
P. micronemus	. . . C	. . A A	. . . . .	. . . . .	. . . . . A	. . . . . A . .	. . . . .	. . . . C	. . . . .	. . . . .	. . . . T	. . . . .	. . . . .
P. pleurotaenia	. . . . .	. . . . .	. . A	. . . . .	. . . . . A	. . . . . A . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. hypophthalmus	. . . C	. . A	. . . . .	. . . . .	. . . . .	. T	. . . . .	. . . . CT	. . . . .	. . . . .	. . AG	. . . . TG	. . . . .
P. gigas	. . . . .	. . . . .	. . A	. . . . .	. . . . . A	. . . . .	. T	. . . . CT	. . . . .	. . . . .	. . AG	. . . . TG	. . . . .
H. leptorhynchus	. . . C	. . . C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . C	. G	. . . . .	. . . G	. T . . T	. . . . .
H. waandersii	. . . C	. . . C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . .	. . . . C	. G	. . . . .	. . . G	. T . . T	. . . . .
H. typus	. . . . .	. . . C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . C	. . . . .	. . . . .	. . . . .	G	. . . . .
S. mandibularis	. . . . .	. . . . .	. . . . C	. . . . .	. . . . . A . . T	GT . . . C . AC .	. . . . .	. . . . C	. . . . .	. . . . . T .	. . . . .	G . . . . T . G . .	. . . . .
L. sinensis	. . . . .	. . . . .	. . . . C	. . . . .	. . . . . A	. A . AA . . AC .	. . . . .	. . . . C . T . . .	. . . T	. . . . . T	. A . . . . . G	G . . . A	. . . . .
C. garua	. . . . .	. . . . .	. . . . C	. . . . .	. . . . .	GA . AAAG . T .	. . . . .	. . . . C	. . . T	. . A . . T . A . .	G . . G	. . . . A	. . . . .
E. vacha	. . . . .	. . . A	. . . . C	. . . . .	. . . . .	TA . . . . AT .	. . . . .	. . . . .	T . . T . . . T . .	. . A . . T	. . . C	. . . . A	. . . . .
S. silondia	. . . . .	. . . . .	. . . . C	. . . . .	. . . . . G	. A . . . C . AC .	. . . . .	. . . . C	. . . T	. . A . . T	. . . . .	. . . . A	. . . . .
L. hexanema	. . . . .	. . . . .	. . . . C	. . . . .	. G . . TG . . . A	. A . AA . . AT .	. . . . .	. . . . C . T . . .	. . . T	. . A . . T	. A	. . . . A	. . . . .
P. brachypopterus	. . . . .	. . . . .	. . TAC	. . . . .	. . . . . G . A	. . . . . C . TT .	. . . . . T . .	. . . GC	. . . . . T . .	. . . . . C . . .	. . . GT . T . . .	G . T . A	. . . . . A . .

Table 2. (continued).

| | | |

Table 2. (continued).

P. polyuranodon Rest of Indonesia	AAAACCCAAA	GGACTTGGCG	GTGTCTCAGA	CCCACCTAGA	GGAGCCTGTT	CTAGAACCGA	TAACCCCCGT	TAAACCTCAC	CACTTCTTGT	T-TTCCCCGC	CTATATACCG	CCGTCGCCAG	CTTACCCTGT				
P. polyuranodon Indo-China	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .				
P. polyuranodon East Kalimantan	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . C	. . . . .	. . . . .	. . . . .	. . . . .				
P. rheophilus	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. djambal	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . C	. . . . .	. . . . . T . . .	. . . . .				
P. bocourti	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. nasutus	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. conchophilus	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. pangasius	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. kunyit Indonesia	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. kunyit Vietnam	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. kunyit North Borneo	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. sanitwongsei	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. larnaudii	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. humeralis	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. nieuwenhuisii	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. kinabatanganensis	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. lithostoma	T	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. macronema	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .				
P. micronemus	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. pleurotaenia	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . C .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. hypophthalmus	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
P. gigas	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
H. leptorhynchus	. . . . .	. . . . .	. G	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . T	. . . . .	. . . . . T . . .	. . . . .				
H. waandersii	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . T	. . . . .	. . . . . T . . .	. . . . .				
H. typus	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .				
S. mandibularis	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . C .	. . . . .	. . . . .	. . . . . T . . .	. . . . . A .				
L. sinensis	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. C	. . . T	. . . . .	. . . . . T . . .	. . . . .				
C. garua	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. C	. T . . T . - . .	. . . . .	. . . . . T . . .	. . . . .				
E. vacha	. . . . .	. . . . .	. . . C	. . . . .	. . . . .	. . . . .	. . . T	. . . . .	. C	. T . . T . - . .	. . . . .	. . . . . T . . .	. . . . .				
S. silondia	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . . T . . .	. . . . .	. C	. T . . T . - . .	. . . . .	. . . . . T . . .	. . . . .				
L. hexanema	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. C	. . . . T . - . .	. . . . .	. . . . . T . . .	. . . . .				
P. brachypopterus	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. T . . . . . - . .	. . . . .	. . . . . T . . .	. . . . .				

Table 2. (continued).

P. polyuranodon Rest of Indonesia	GAAGGCCT-A	GCAGTAAGCA	AAATGGGCTC	GCCCAAAAAC	GTCAGGTCGA	GGTGTAGCGC	ACGAAGTGGG	AAGAAATGGG	CTACATTTTC	TGCA-GC-AG	AATACT-ACG	GAC-GGCACC	CTGAAACA-A
P. polyuranodon Indo-China	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A.	....T. . . .	A.	. . . . .
P. polyuranodon East Kalimantan	. . . . .T. . . .	AG.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A.	....T. . . .	A.	. . . . .
P. rheophilus	. . . . .	. . . . .	..G. . . .C.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.AG. .AG. . .	....T. . . .	A.T. . .T. .T	. . . . .
P. djambal	A.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .A. . . .	....T. . . .	A.	. . . . .
P. bocourti	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .A. . . .	....T. . . .	A.	. . . . .
P. nasutus	. . . . .	A.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .A. . . .	....T. . . .	A.T.	. . . . .
P. conchophilus	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .AT. . .	....T. . . .	A.T.	. . . . .
P. pangasius	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .A. . . .	....T. . . .	A. . . . . T	. . . . .
P. kunyit Indonesia	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .A. . . .	....T. . . .	A.	. . . . .
P. kunyit Vietnam	. . . . .	. . . . .	. . . . .	A.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.AT. .A. . . .	....T. . . .	A.	. . . . .
P. kunyit North Borneo	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	....G. . . .	. . . . .	. . . . .	.A. . .A. . . .	....T. . . .	A.	. . . . .
P. sanitwongsei	. . . . .	. . . . .	..G.	A.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .A. . . .	....T. . . .	A.T. . . . . T	. . . . .
P. larnaudii	. . . . .	.T.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .A. . . .	....T. . . .	A.T. . .TG.T	. . . . .
P. humeralis	. . . . . C. .	A.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .A. . . .	....T. . . .	A.	. . . . .
P. nieuwenhuisii	. . . . . C. .	A.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .A. . . .	....T. . . .	A.	. . . . .
P. kinabatanganensis	. . . . . .T.	A.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .A. . . .	..C.T. . . .	A. . . . . T	. . . . .
P. lithostoma	. . . . . CT.	A.	. . . . .	A.	. . . . .	. . . . .	. . . . . A	. . . . .	. . . . .	.A. . .A. . . .	....T. . . .	A.T. . . . . TA	. . . . .
P. macronema	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A.	....T. . . .	A.	. . . . .
P. micronemus	. . . . .T. . . .	AT.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .A. . . .	....T.T. . .	A.T.	. . . . .
P. pleurotaenia	. . . . .T.A. .	AT.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .A. . . .	....T.T. . .	A.T.	. . . . .
P. hypophthalmus	. . . . .	. . . . .	..G.	....C.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .AT. . .	....T. . . .	A.T.	. . . . .
P. gigas	. . . . .T.C. .	A.	. . . . . A	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .AT. . .	....T. . . .	A.T.	. . . . .
H. leptorhynchus	. . . . .	.T.	....T. . .C.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .AT. . .	....T. . . .	A.T.	. . . . .
H. waandersii	. . . . .	.T.	....T. . .C.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.A. . .AT. . .	....T. . . .	A.T.	. . . . .
H. typus	. . . . .	AT.	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	.AT. .A. . . .	....T. . . .	A.T.	. . . . .
S. mandibularis	. . . . .AT												
L. sinensis	. . . . .												
C. garua	. . . . .												
E. vacha	. . . . .T.												
S. silondia	. . . . .												
L. hexanema	. . . . .												
P. brachypopterus	. . . . .												

Table 2. (continued).

P. polyuranodon Rest of Indonesia	GTGCCCGAAG	GTGGATTTAG	TAGTAAAAAG	CAAATAGAGA	GTCCTTTTGA	ATTAGGCTCT	GAGACGCGCA	CACACCGCCC	GTCACTC
P. polyuranodon Indo-China	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. polyuranodon East Kalimantan	. . . . . T	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. rheophilus	. . . . . T	. . . . .	. . . . .	 C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. djambal	. . . . .	. . . . .	. . . . .	 C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. bocourti	. . . . .	. . . . .	. . . . .	 C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. nasutus	A . . . . T	. . . . .	. . . . .	 C	. . . T	. . . . .	. . . . .	. . . . .	. . . . .
P. conchophilus	A . . . . T	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. pangasius	. . . T . T	. . . . .	. . . . .	 C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. kunyit Indonesia	. . . . . T	. . . . .	. . . . .	 C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. kunyit Vietnam	. . . . . T	. . . . .	. . . . .	 C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. kunyit North Borneo	. . . . . T	. . . . .	. . . . .	 C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. sanitwongsei	. . . . . T	. . . . .	. . . . .	 C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. larnaudii	. . A . . T	. . . . .	. . . . .	 C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. humeralis	. . . . .	. . . . .	C	T C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. nieuwenhuisii	. . . . .	. . . . .	C	 C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. kinabatanganensis	. . . . . T	. . . . .	. . . . .	. . . . . T	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. lithostoma	. . . . . T	. . . . .	. . . . .	 C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. macronema	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. micronemus	A . . . . T	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. pleurotaenia	A . . . . T	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. hypophthalmus	A . . . . T	. . . . .	. . . . .	. . . . . T	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
P. gigas	. . . . . T	. . . . .	. . . . .	. . . . . T	. . A	. . . . .	. . . . .	. . . . .	. . . . .
H. leptorhynchus	A . . . . T	. . . . .	. . . . .	 C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
H. waandersii	A . . . . T	. . . . .	. . . . .	 C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
H. typus	A . . . . T	. . . . .	. . . . .	 C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .

(Sumatra and Kalimantan), North Borneo (Sabah), and Indo-China (Vietnam). Within *P. kunyit*, the average genetic distances between populations from North Borneo and groups from Indonesia (d = 0.007) and Indo-China (d = 0.0097) or among the two latter (d = 0.003) correspond respectively to those estimated between *P. macronema* and *P. polyuranodon* in Vietnam (d = 0.004), *P. djambal* and *P. bocourti* (d = 0.0055), *Helicophagus waandersii* and *H. leptorhynchus* (d = 0.004).

Putative new *Pangasius* species inside *P. polyuranodon* and *P. kunyit* will be examined in further studies, but for the present contribution, they will be considered as genetically distant populations. They will be respectively labelled as POL 1 (populations of *P. polyuranodon* from Indonesia except East Kalimantan), POL 2 (populations of East Kalimantan), POL 3 (populations of Indo-China: Mekong and Chao Phraya Rivers), KUN 1 (populations of *P. kunyit* from Indonesia), KUN 2 (populations of Indo-China), KUN 3 (populations of North Borneo).

Finally, in a second step, corrected Kimura's genetic distances (below diagonal) and absolute substitution/insertion/deletion number (above diagonal) are presented for each possible couple of species (Table 3). Pangasiid species are well differentiated with genetic distance comprised between 0.0042 and 0.0561. The most closely related species are *H. waandersii* and *H. leptorhynchus* (d = 0.0042), *P. djambal* and *P. bocourti* (d = 0.0055), *P. nieuwenhuisii* and *P. humeralis* (d = 0.0056), and the most differentiated are *P. gigas* and *P. rheophilus* (d = 0.0561).

A significant linear correlation between Kimura's distances and the number of transitions and transversions per site suggested that few multiple substitutions have occurred at most nucleotide positions and are far from being saturated. This indicated that interspecific genetic distances among pangasiids are likely within the range to be phylogenetically instructive (Lydeard and Roe 1997).

The nearest neighbour dendrogram was inferred from the corrected Kimura's genetic distance pairwise matrix among taxa (Fig. 1). Four monophyletic groups were validated by the bootstrapping tests: group 1 (bootstrap 84%) including *Pangasianodon hypophthalmus* and *P. gigas*; group 2 (bootstrap 92%) composed of *Pteropangasius micronemus* and *P. pleurotaenia*; group 3 (bootstrap 80%) containing *Helicophagus waandersii*, *H. leptorhynchus*, *H. typus*; and group 4 (bootstrap 100%) with *P. humeralis*, *P. nieuwenhuisii*, *P. polyuranodon*, *P. macronema*. Besides groups 3 and 4, seven highly differentiated species are branched in the internal part of the tree, namely *P. lithostoma*, *P. nasutus*, *P. larnaudii*, *P. sanitwongsei*, *P. rheophilus*, *P. conchophilus*, and *P. kinabatanganensis*. In this part of the tree, the poor resolution of branching order of most internal nodes does not allow resolution of the phylogeny, although some pairs of related species could be clearly evidenced, for example *P. djambal* and *P. bocourti* (bootstrap 100%), *P. kunyit* and *P. pangasius* (bootstrap 50%). This lack of resolution in the *Pangasius* (*Pangasius*) subgenus was also noted for allozymes and cytochrome b phylogenies (Pouyaud *et al*. 2000) and was interpreted to be an expected signature of an intense radiation in a short period, too brief to allow for the accumulation of synapomorphies (Johns and Avise 1998; Martin and Bermingham 1998). Overall, the topology of the 12S rDNA phylogeny is similar to those obtained with allozymes and cytochrome b (Pouyaud *et al*. 2000).

Genetic Interrelationships between Pangasiidae and Schilbeidae

Five hundred and twenty seven nucleotides encoding the first half of the 12S rDNA gene were aligned between seven schilbeids and all the pangasiids (Table 2). Hundred and thirthy one sites (25%) were polymorphic. Corrected Kimura's genetic distances (below diagonal) and transition/transversion ratios (above diagonal) were calculated between the schilbeids and five pangasiids (*H. waandersii*, *Pangasius nasutus*, *P. polyuranodon*, *Pteropangasius pleurotaenia* and *Pangasianodon hypophthalmus*) representing the hypothesised genera (Table 4). The nearest neighbour dendrogram was inferred from the corrected Kimura's genetic distance pairwise matrix among species (Fig. 2). The topology of the resulting tree separates Schilbeidae from Pangasiidae.

The same results were obtained when including all the pangasiid species, but the corresponding tree is not given. Schilbeidae are characterised by three synapomorphic substitutions (respectively in nucleotide positions 90, 275, and 277). However, the monophyly of Schilbeidae is not supported by high bootstrap value (approximately 50%) due to the existence of three distinct lineages separated by huge genetic divergence. As an example, the low transition/transversion ratios calculated for the 12S rDNA sequences of *Schilbe mandibularis* and *Pseudeutropius brachypopterus* confirm that nucleotidic substitutions are saturated. Nevertheless, a monophyletic group is supported by a strong bootstrap value (100%) in the schilbeids. This highly

Table 3. Corrected Kimura's genetic distances between Pangasiidae (below diagonal) and absolute number of substitution/insertion/deletion for each pairwise comparison (above diagonal).

Species	pol.1	pol.2	pol.3	rhe	dja	boc	nas	con	pan	kun.1	kun.2	kun.3	san
P. polyuranodon Rest of Indonesia (1)	------	10	20	30	22	20	26	24	21	17	19	21	25
P. polyuranodon Indo-China (2)	0.0126	------	22	29	25	23	26	24	22	20	22	22	28
P. polyuranodon East Borneo (3)	0.0253	0.0253	------	38	33	31	31	33	31	27	29	10	35
P. rheophilus	0.0413	0.0384	0.0502	------	31	29	23	18	20	22	23	23	27
P. djambal	0.0268	0.0326	0.0428	0.0384	------	4	22	23	23	19	21	22	23
P. bocourti	0.0240	0.0297	0.0199	0.0155	0.0055	------	20	21	21	17	19	20	21
P. nasutus	0.0340	0.0354	0.0411	0.0283	0.0268	0.0239	------	11	17	15	17	18	20
P. conchophilus	0.0311	0.0325	0.0442	0.0240	0.0282	0.0253	0.0182	------	19	15	17	16	23
P. pangasius	0.0326	0.0297	0.0413	0.0268	0.0282	0.0254	0.0211	0.0239	------	10	12	11	19
P. kunyit Indonesia (1)	0.0239	0.0268	0.0355	0.0297	0.0225	0.0197	0.0182	0.0182	0.0140	------	2	5	19
P. kunyit Vietnam (2)	0.0268	0.0296	0.0384	0.0311	0.0253	0.0225	0.0211	0.0211	0.0168	0.0028	------	7	19
P. kunyit North Borneo (3)	0.0297	0.0296	0.0413	0.0311	0.0268	0.0239	0.0225	0.0197	0.0154	0.0070	0.0097	------	22
P. sanitwongsei	0.0341	0.0370	0.0458	0.0356	0.0269	0.0240	0.0268	0.0312	0.0253	0.0254	0.0254	0.0297	------
P. larnaudii	0.0369	0.0398	0.0472	0.0312	0.0340	0.0311	0.0251	0.0254	0.0268	0.0182	0.0211	0.0225	0.0297
P. humeralis	0.0297	0.0268	0.0398	0.0326	0.0355	0.0326	0.0297	0.0297	0.0311	0.0251	0.0282	0.0296	0.0428
P. nieuwenhuisii	0.0297	0.0268	0.0370	0.0326	0.0355	0.0326	0.0297	0.0297	0.0311	0.0253	0.0282	0.0296	0.0428
P. kinabatanganensis	0.0311	0.0326	0.0384	0.0341	0.0282	0.0254	0.0254	0.0297	0.0253	0.0253	0.0282	0.0296	0.0326
P. lithostoma	0.0503	0.0533	0.0563	0.0460	0.0415	0.0386	0.0298	0.0386	0.0370	0.0298	0.0298	0.0341	0.0342
P. macronema	0.0084	0.0042	0.0210	0.0340	0.0282	0.0254	0.0111	0.0282	0.0254	0.0225	0.0253	0.0253	0.0326
P. micronemus	0.0385	0.0428	0.0444	0.0371	0.0370	0.0341	0.0255	0.0269	0.0370	0.0326	0.0355	0.0370	0.0371
P. pleurotaenia	0.0199	0.0442	0.0458	0.0415	0.0384	0.0355	0.0269	0.0312	0.0384	0.0341	0.0370	0.0384	0.0400
P. hypophthalmus	0.0443	0.0428	0.0517	0.0458	0.0428	0.0399	0.0384	0.0370	0.0427	0.0325	0.0354	0.0397	0.0414
P. gigas	0.0547	0.0576	0.0592	0.0561	0.0472	0.0443	0.0458	0.0458	0.0516	0.0384	0.0413	0.0457	0.0503
H. leptorhynchus	0.0502	0.0532	0.0562	0.0487	0.0458	0.0428	0.0370	0.0370	0.0384	0.0268	0.0268	0.0311	0.0414
H. waandersii	0.0517	0.0546	0.0577	0.0501	0.0472	0.0443	0.0355	0.0385	0.0398	0.0282	0.0282	0.0326	0.0429
H. typus	0.0370	0.0369	0.0443	0.0313	0.0327	0.0298	0.0226	0.0255	0.0254	0.0211	0.0211	0.0239	0.0356

pol = *Pangasius polyuranodon*, rhe = *P. rheophilus*, dja = *P. djambal*, boc = *P. bocourti*, nas = *P. nasutus*, con = *P. conchophilus*, pan = *P. pangasius*, kun =*P. kunyit*, san = *P. sanitwongsei*, lar = *P. larnaudii*, hum = *P. humeralis*, nie = *P. nieuwenhuisii*, kin = *P. kinabatanganensis*, lit = *P. lithostoma*, mac = *P. macronema*, mic = *P. micronemus*, ple = *P. pleurotaenia*, hyp = *P. hypophthalmus*, gig = *P. gigas*, lep = *Helicophagus leptorhynchus*, waa = *H. waandersii*, typ = *H. typus*

Table 3. (continued).

Species	lar	hum	nie	kin	lit	mac	mic	pie	hyp	gig	lep	waa	typ
P. polyuranodon Indonesia (1)	27	22	22	24	38	7	32	32	32	38	35	36	27
P. polyuranodon Indochina (2)	30	21	21	26	38	3	34	33	32	41	38	39	28
P. polyuranodon East Borneo (3)	36	31	29	31	43	16	36	36	39	43	41	42	34
P. rheophilus	24	25	25	27	35	26	32	34	34	39	35	36	24
P. djambal	28	29	29	25	34	22	30	30	33	36	35	36	27
P. bocourti	26	27	27	23	32	20	28	28	32	34	33	34	25
P. nasutus	19	24	24	22	26	23	23	23	30	34	28	27	19
P. conchophilus	19	24	24	25	31	21	24	26	28	33	28	27	21
P. pangasius	20	23	23	20	28	19	31	33	31	35	27	28	19
P. kunyit Indonesia (1)	14	19	18	20	23	17	27	28	24	27	19	20	16
P. kunyit Vietnam (2)	16	21	21	22	23	19	30	30	26	29	19	20	16
P. kunyit North Borneo (3)	17	22	22	23	26	19	31	31	29	32	22	23	18
P. sanitwongsei	21	32	32	26	27	25	32	33	39	36	30	31	27
P. larnaudii	------	29	29	25	30	27	32	30	29	33	23	26	23
P. humeralis	0.0383	------	4	20	35	17	34	31	34	35	43	45	26
P. nieuwenhuisii	0.0384	0.0056	------	20	35	18	35	33	36	37	36	38	27
P. kinabatanganensis	0.0311	0.0268	0.0268	------	32	23	35	31	31	37	37	38	26
P. lithostoma	0.0385	0.0473	0.0474	0.0429	------	38	35	35	37	36	33	34	27
P. macronema	0.0355	0.0255	0.0225	0.0283	0.0488	------	32	31	29	38	35	36	25
P. micronemus	0.0371	0.0414	0.0414	0.0400	0.0401	0.0384	------	11	33	34	35	35	23
P. pleurotaenia	0.0356	0.0385	0.0414	0.0356	0.0415	0.0398	0.0140	-------	32	28	36	38	24
P. hypophthalmus	0.0384	0.0457	0.0486	0.0398	0.0502	0.0384	0.0399	0.0413	------	23	33	34	28
P. gigas	0.0458	0.0487	0.0517	0.0502	0.0503	0.0532	0.0415	0.0341	0.0312	------	37	38	34
H. leptorhynchus	0.0312	0.0531	0.0532	0.0502	0.0429	0.0487	0.0430	0.0474	0.0458	0.0531	------	3	22
H. waandersii	0.0326	0.0546	0.0546	0.0516	0.0444	0.0501	0.0444	0.0488	0.0472	0.0545	0.0042	------	23
H. typus	0.0312	0.0355	0.0355	0.0326	0.0327	0.0326	0.0269	0.0298	0.0398	0.0472	0.0298	0.0312	------

pol = *Pangasius polyuranodon*, rhe = *P. rheophilus*, dja = *P. djambal*, boc = *P. bocourti*, nas = *P. nasutus*, con = *P. conchophilus*, pan = *P. pangasius*, kun = *P. kunyit*, san = *P. sanitwongsei*, lar = *P. larnaudii*, hum = *P. humeralis*, nie = *P. nieuwenhuisii*, kin = *P. kinabatanganensis*, lit = *P. lithostoma*, mac = *P. macronema*, mic = *P. micronemus*, ple = *P. pleurotaenia*, hyp = *P. hypophthalmus*, gig = *P. gigas*, lep = *Helicophagus leptorhynchus*, waa = *H. waandersii*, typ = *H. typus*

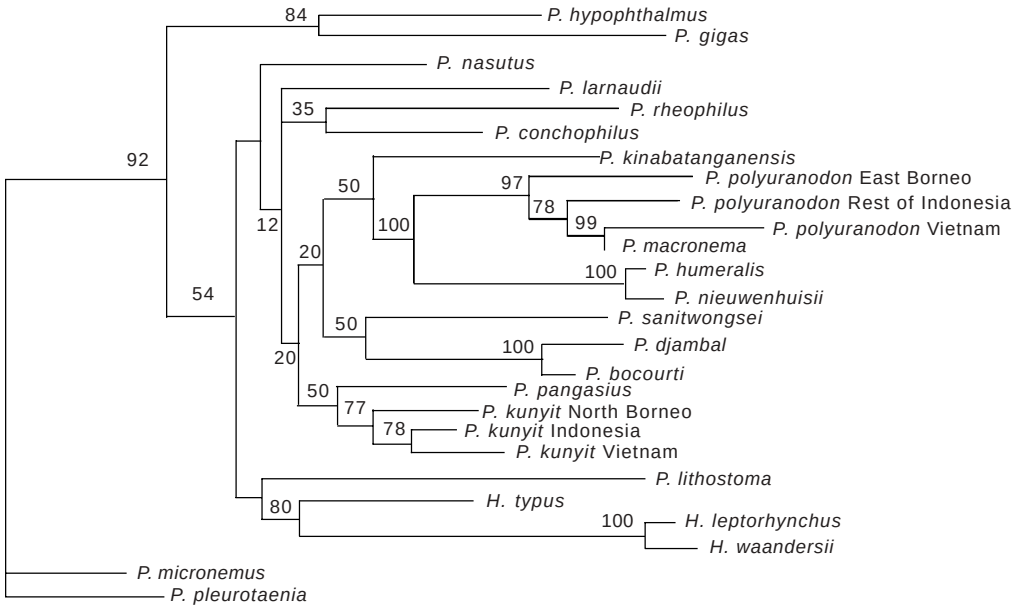

Fig. 1. The nearest neighbour dendrogam for 12S rDNA gene within Pangasiidae. Numbers next to node show percentage group occurrence found with the 1000 bootstrapped trees.

differentiated group, characterised by three synapomorphic substitutions (positions 245, 482, 488) and assembling *Laides* with *Clupisoma*, *Eutropiichthys* and *Silonia* clearly demonstrates that the genus *Laides* cannot be included in the Pangasiidae. Moreover, when considering the average genetic distances within Pangasiidae as a reference (Table 4), Kimura's genetic distances computed within Schilbeidae suggest the possible occurrence of several distinct families. Actually, equivalent genetic divergences are found between the two lineage of Asian schilbeids ($0.0863 < d_{Kim.} < 0.1040$ between *P. brachypopterus* and others) or between both lineage of Asian schilbeids and lineage of African schilbeids ($d_{Kim.} = 0.1028$ and $0.0980 < d_{Kim.} < 0.1167$, respectively).

Evolutionary Process Based on Speciation Peaks

The distribution pattern of pairwise Kimura-corrected genetic distance on the 12S rDNA gene was studied for all Pangasiidae and Schilbeidae species (Fig. 3). Three distinct modes were observed, corresponding respectively to interfamily, intergenus, and intragenus assignation of pairwise comparisons. The first mode, distributed between 0.0575 and 0.1025, corresponds to the most ancient radiation between Schilbeidae and Pangasiidae. The second mode, distributed between the values of 0.0175 and 0.065, corresponds to the radiation between the genera of Pangasiidae and among some genera of the Schilbeidae, except *Pseudeutropius* and *Schilbe*. The radiation of *Pseudeutropius* and *Schilbe* is more ancient (between 0.0875 and 0.1175) and overlaps with the interfamily radiation peak (right part) suggesting again that both genera probably belong to distinct families. Finally, the third peak, between 0.001 and 0.05, corresponds especially to the radiation within the different genera of both families. The right part of this peak, largely superposed with the intergenus mode between 0.03 and 0.05 mainly concerns an earlier radiation within *Pteropangasius*, *Helicophagus*, *Pangasianodon*, and *Clupisoma*. Except for *P. lithostoma*, *P. polyuranodon*, and *P. kinabatanganensis*, the radiation within *Pangasius* is more recent (between 0.002 and 0.03).

Systematic Position of the Genus *Laides*

Molecular analyses clearly showed that Pangasiidae and Schilbeidae are in two distinct families. They also reveal in the Schilbeidae, the existence of different lineages characterised by a large genetic divergence. The genus *Laides* forms a monophyletic assemblage with the genera *Eutropiichthys*, *Clupisoma* and *Silonia*, and this definitely indicates that it belongs to the Schilbeidae. The average genetic distances estimated among species in this group are equivalent to those observed in Pangasiidae and suggest that both distant taxa *Schilbe* and *Pseudeutropius* may belong to distinct families. The important genetic divergence noted in Schilbeidae is also enforced by their important morphological diversity and their

Table 4. Corrected Kimura's genetic distances between 5 species of Pangasiidae and 7 species of Schilbeidae (below diagonal) and absolute number of substitution (transition/ transversion) for each pairwise comparison (below diagonal).

Species	pol	nas	ple	hyp	waa	man	sin.	gar	vac	sil	hex	bra
Pangasius (Pangasius) polyuranodon	-----	9/3	10/6	16/4	17/7	20/16	25/9	25/10	25/15	26/10	25/11	24/10
Pangasius (Pangasius) nasutus	0.0236	-----	11/5	16/2	14/5	20/13	25/6	26/8	28/12	26/7	28/8	20/10
Pangasius (Pteropangasius) pleurotaenia	0.0317	0.0296	-----	17/3	21/7	21/15	25/10	25/12	24/16	22/11	26/12	23/14
Pangasius (Pangasianodon) hypophthalmus	0.0397	0.0377	0.0396	-----	20/4	25/16	33/7	31/9	29/13	30/8	32/9	24/10
Helicophagus waandersii	0.0480	0.0377	0.0562	0.0499	-----	29/19	38/10	27/14	30/18	29/13	32/14	22/12
Schilbe mandibularis	0.0739	0.0717	0.0716	0.0848	0.1005	-----	30/17	31/19	32/23	28/17	35/17	28/20
Clupisoma sinensis	0.0693	0.0627	0.0693	0.0820	0.0867	0.0980	-----	18/2	23/6	17/1	12/2	32/10
Clupisoma garua	0.0736	0.0692	0.0737	0.0821	0.0846	0.1049	0.0395	-----	18/8	16/3	16/4	32/12
Eutropiichthys vacha	0.0827	0.0824	0.0805	0.0870	0.1006	0.1167	0.0583	0.0522	-----	17/5	23/6	36/14
Silonia silondia	0.0735	0.0668	0.0650	0.0777	0.0866	0.0936	0.0355	0.0376	0.0437	-----	18/1	34/9
Laides hexanema	0.0736	0.0733	0.0757	0.0842	0.0954	0.1114	0.0274	0.0396	0.0582	0.0375	-----	34/10
Pseudotropius brachypopterus	0.0692	0.0629	0.0737	0.0715	0.0714	0.1028	0.0863	0.0905	0.1040	0.0880	0.0904	-----

pol = *Pangasius (Pangasius) polyuranodon*, nas = *Pangasius (Pangasius) nasutus*, ple = *Pangasius (Pteropangasius) pleurotaenia*, hyp = *Pangasius (Pangasianodon) hypophthalmus*, waa = *Helicophagus waandersii*, man = *Schilbe mandibularis*, sin = *Clupisoma sinensis*, gar = *Clupisoma garua*, vac = *Eutropiichthys vacha*, sil = *Silonia silondia*, hex = *Laides hexanema*, bra = *Pseudotropius brachypopterus*.

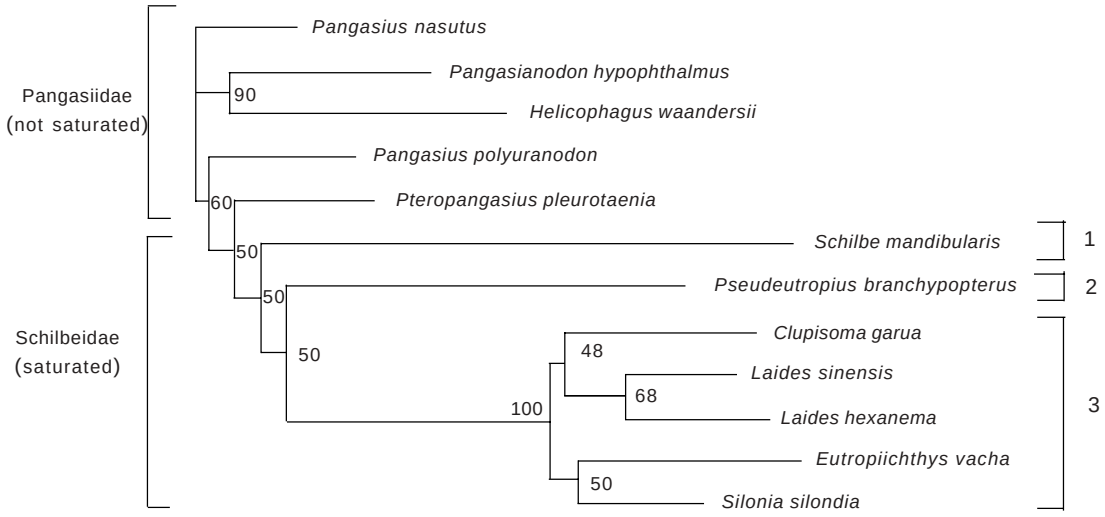

Fig. 2. The nearest neighbour dendrogam from partial 12S rDNA gene of Pangasiidae and Schilbeidae. Numbers next to node show the percentage group occurrence found with the 1000 bootstrapped trees.

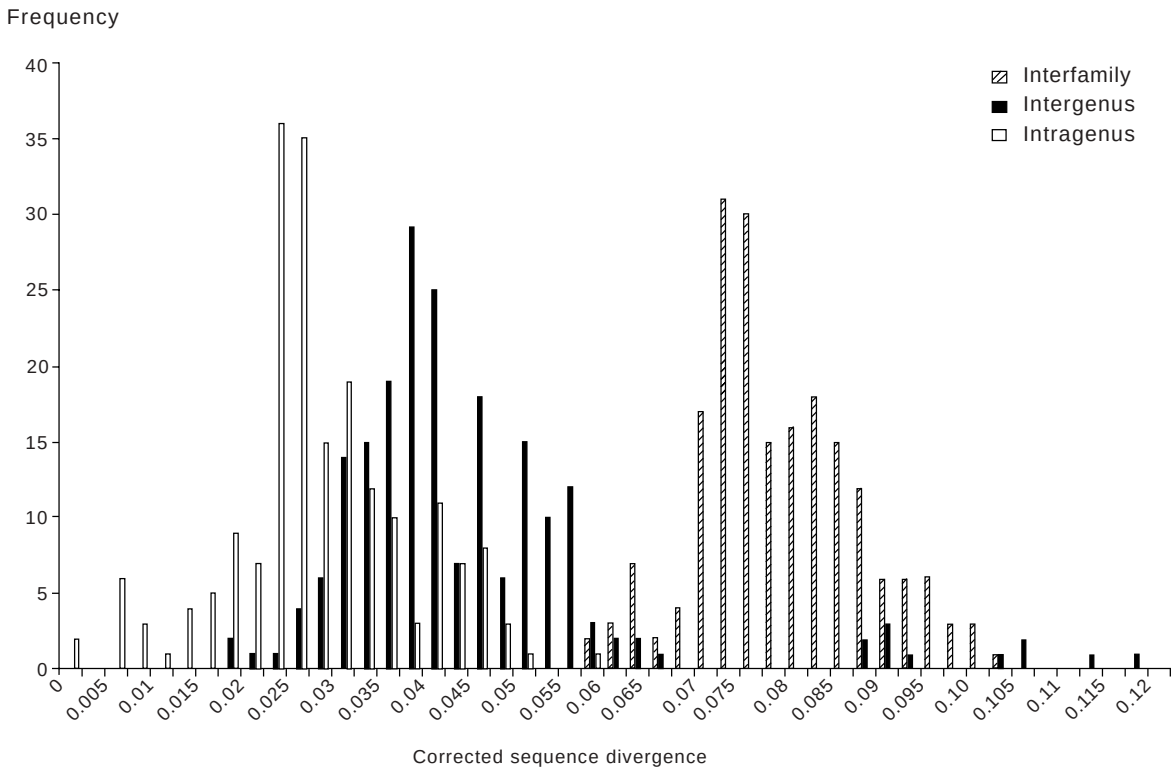

Fig. 3. Distribution of corrected Kimura's genetic distances at all positions respectively for intragenus, intergenus, and interfamily pairwise comparisons.

wide distribution through Asia and Africa, which suggests a long evolutionary process since the emergence of this family.

As demonstrated above, Pangasiidae and Schilbeidae probably stem from a common ancestor. At present, Schilbeidae mainly occurs in Africa and on the Indian subcontinent, while Pangasiidae are mainly found in Southeast Asia. The molecular phylogenies obtained from these groups always revealed important genetic differentiation. Referring to the abundant literature concerning approximate molecular clock/evolutionary time calibrations available on mitochondrial genes, an estimation of time divergence between both families or within them can be given below.

It is accepted that the overall rate of nucleotide substitutions for the complete mitochondrial genome may be 0.5-2% per million years depending on the various selective constraints occurring on the different gene families (Naylor *et al.* 1995; Zardoya and Meyer 1996; Kocher and Carlton 1997). The small ribosomal subunit (12S) gene is characterised by the lowest rate of evolution in the mitochondrial DNA (between 0.5 and 1% per million years) due to severe structural or/and functional constraints rather than a depressed mutation rate (Simon *et al.* 1994). By contrast, cytochrome b evolves faster (between 1 and 2% per million years) due to silent substitutions mainly occurring in third position of the codon (Meyer 1993). Pouyaud *et al.* (2000) estimated an average time of divergence between the Schilbeidae and Pangasiidae around 20 million years by using a rate of cytochrome b evolution proposed for marine fishes (1-1.2% sequence divergence per million years) (Bermingham *et al.* 1997). An equivalent estimation between 14 and 20 million years ago (mya) is estimated with 12s rDNA when using a rate of 0.5% per million years. Therefore, we consider that the separation between Schilbeidae and Pangasiidae occurred in the Early Miocene during the opening of the Red Sea and achievement of tectonic uplifts in Northern Thailand.

By using the same rate of evolution as above for 12S rDNA, the average time of divergence between the four pangasiids genera is ≈ 7-11 mya. This estimation corresponds to the Middle and Late Miocene. More recently (≈ 5-8 mya), a new episode of speciation marked by an explosive radiation is observed in the genus *Pangasius*. Similar dating was estimated with other molecular markers (≈ 8-10 mya with cytochrome b and ≈ 9 mya with allozymes) by Pouyaud *et al.* (2000). This adaptative radiation extended until the Late Pleistocene (1-4 mya, referring to the split between *Pteropangasius* species, *H. waandersii* and *H. leptorhynchus*, *P. humeralis* and *P. nieuwenhuisii*, *P. polyuranodon* and *P. macronema*, *P. bocourti* and *P. djambal* or different groups of *P. kunyit*).

Speciation Process

The divergence time assessed with molecular tools (Fig. 4) agrees with the hypothesis proposed by Vidthayanon (1993), mentioning that the dispersal of Pangasiidae cannot be merely explained by recent connections of river systems, but may be traced back to the early Tertiary or even earlier times when the present-day continental and insular sections constituted a land mass known as the "Indonesian-Cathaysian land". Under these conditions, cumulative fluctuations of sea water levels coupled with important tectonic movements during millions of years undoubtedly have fashioned the pangasiids leading to the notable morphological and taxonomic diversity known at present (De Beaufort 1951; Taki 1978; Rainboth 1991).

The large river basins in Asian continent (the Mekong and Chao Phraya) and the island of western Indonesia (the Kapuas and Mahakam in Kalimantan), which possess the highest species diversity, have certainly played a major role in terms of refuge zones during the past (Roberts 1989; Kottelat 1995) when marine transgression occurred. Refugee zones represent sites with a peculiar flow regime and vegetation linking to the tropical forest. This resulted in a vicariant speciation process. A good example illustrating this scenario is the comparison of pangasiid fauna between continental Asia and the Indo-Malay Archipelago revealing the existence of related species with allopatric distribution. This is respectively true for *H. leptorhynchus* and *H. waandersii*, *P. conchophilus* and *P. nasutus*, *P. bocourti* and *P. djambal* or between population groups within *P. kunyit* and *P. polyuranodon*.

As a consequence of human activities and heavy exploitation, many species in this family are presently being threatened with extinction. The World Conservation Union (IUCN) already lists some species as endangered, e.g. *Pangasianodon gigas* Chevey, 1930 and *Pangasius sanitwongsei* Smith 1931. Thus, the extraordinary diversity and distribution pattern of the Pangasiidae based on a long evolutionary process is now being rapidly modified under human threats. The basic information derived from this study is very important in understanding the relationships, biology and hence the study of the aquaculture potential of species, the improvement of their seed production and growth performance.

CONCLUSION

The present study recognised four genera, i.e. *Pteropangasius* Fowler, 1937; *Helicophagus* Bleeker, 1858; *Pangasianodon* Chevey, 1930; and *Pangasius* Valenciennes, 1840. *Pangasius* is more derive than other genera. The oldest genus may already have existed when the Asian mainland was still connected to the islands in the southern part, about 20 million years ago (mya), during the Miocene glaciation.

The separation between the Schilbeidae and Pangasiidae occurred in the Early Miocene. Diver-

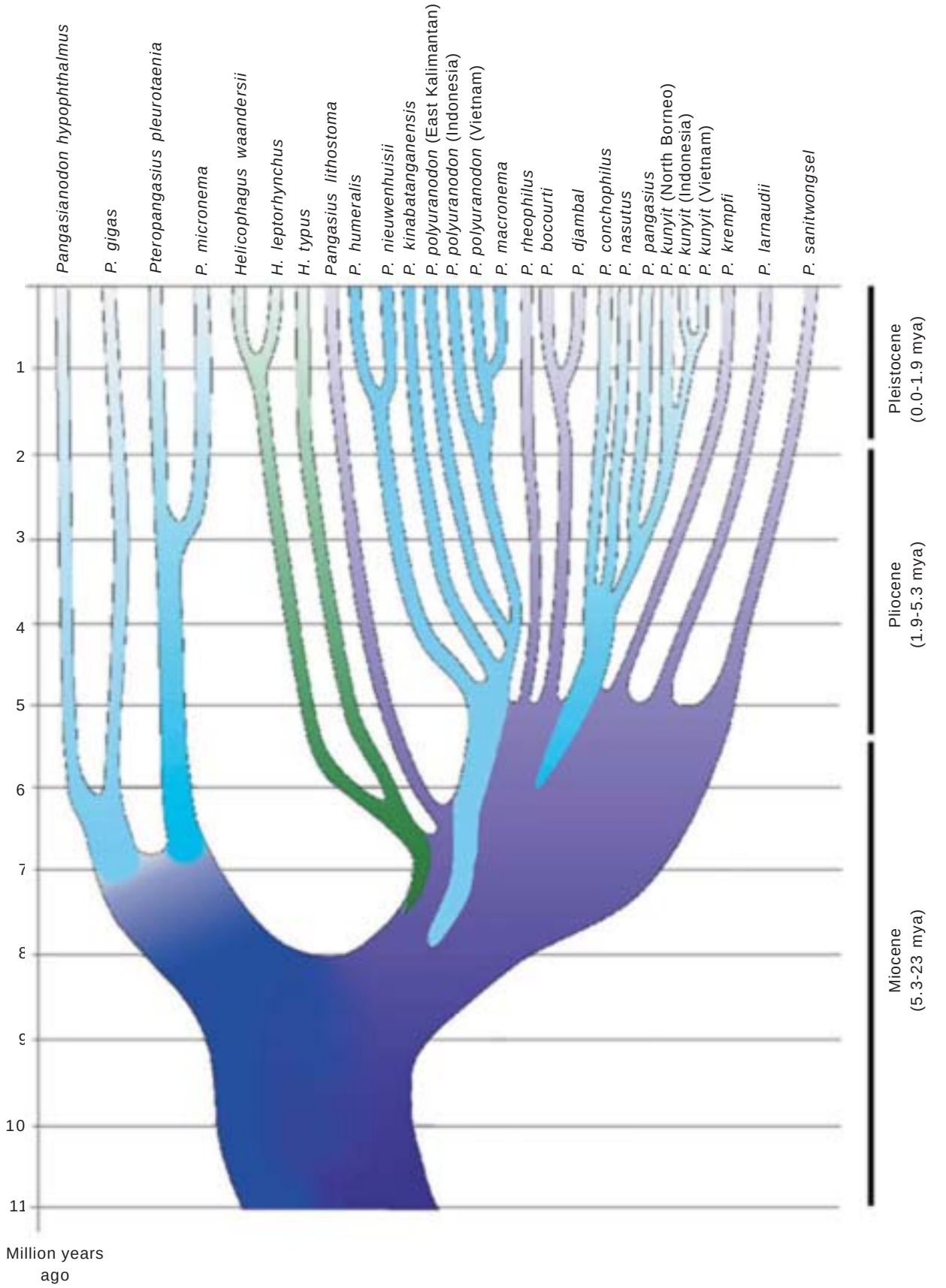

Fig. 4. Evolution within the Pangasiidae as assessed with molecular tools. Speciation has occurred in the Late Miocene for the four pangasiid genera followed by explosive radiation within in the genera in the Early Pliocene and extended to the Late Miocene. Geologic time scale adapted from Ingersoll and Ernst (1987).

gence between the four pangasiids genera dates to about 7-11 mya. A new episode of speciation (about 5-8 mya), marked by an explosive radiation, is observed in the genus *Pangasius*. This adaptive radiation extended until the Late Pliocene (1-4 mya).

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